Reciprocal post-translational regulation of renal 1 α - and 24-hydroxylases of 25-hydroxyvitamin D₃ by phosphorylation of ferredoxin

mRNA-directed cell-free synthesis and immunoisolation of ferredoxin

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We have used a cell-free rabbit reticulocyte translational system programmed with polyadenylated [poly(A)⁺] RNA prepared from chick kidney tissue to study the synthesis of nascent ferredoxin, a class of iron-sulphur-containing proteins functional in the renal mitochondrial 1 α - and 24-hydroxylases of 25-hydroxyvitamin D₃. The synthesis of ferredoxin was monitored by determining [³⁵S]methionine incorporation into ferredoxin and quantified by SDS/PAGE and autoradiography after immunoprecipitation from the total translation products. Compared with normal controls, vitamin D deprivation caused a significant increase in the net synthesis of nascent ferredoxin with an *M_r* of 12000–13000. [³H]Orotate incorporation as uridine into kidney poly(A)⁺ RNA was stimulated by aminophylline, a potent inducer of 25-hydroxyvitamin D₃ 24-hydroxylase; however, the amount of nascent ferredoxin synthesis was the same as in normal controls. Also, partially purified chick kidney mitochondrial cyclic AMP-stimulated protein kinase catalysed the phosphorylation of ferredoxin *in vitro*. The catalytic activity of the ferredoxin in 1 α - and 24-hydroxylations of 25-hydroxyvitamin D₃ in reconstituted systems consisting of cytochrome *P*-450 and ferredoxin reductase was altered with ferredoxin phosphorylation. The phosphorylation caused inhibition of the 1 α -hydroxylase activity while at the same time it stimulated the 24-hydroxylase. Authentic 1 α ,25- and 24,25-dihydroxyvitamin D₃ and 25-hydroxyvitamin D₃ were used as standards to monitor the separation of the enzymic products by h.p.l.c. using methanol/water (4:1, v/v) as solvent. These results indicate that, in the absence of vitamin D or its metabolites in the deficient state, the synthesis of ferredoxin necessary for the 1 α -hydroxylase is accentuated, whereas the stimulation of the 24-hydroxylase requires the phosphorylation of existing ferredoxin without a net gain in its synthesis. This would suggest a post-translational regulation of the 1 α - and 24-hydroxylases. A model delineating the various aspects of this study is presented.

INTRODUCTION

In kidney mitochondria 25-hydroxyvitamin D₃ [25(OH)D₃] is a substrate for two hydroxylation reactions that yield either 1 α ,25- or 24,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃ and 24,25(OH)₂D₃]. Under conditions of vitamin D deficiency, a mitochondrial enzyme designated '25(OH)D₃ 1 α -hydroxylase,' which catalyses the production of 1 α ,25(OH)₂D₃, is the predominant activity in the kidney. The 1 α -hydroxylase is a mono-oxygenase complex composed of NADPH:ferredoxin reductase, ferredoxin and cytochrome *P*-450. The cytochrome functions as a terminal oxidase, and its reduction by NADPH is an essential step in the catalytic activation of molecular oxygen, leading to the production of 1 α ,25(OH)₂D₃ [1–3]. Under normal physiological conditions the synthesis of 1 α ,25(OH)₂D₃ is minimal, but another kidney mitochondrial enzyme, designated '25(OH)D₃ 24-hydroxylase', catalyses the transformation of 25(OH)D₃ to 24,25(OH)₂D₃ [4,5]. The 24-hydroxylase is also a cytochrome *P*-450-catalysed mono-oxygenase complex

[6,7]. We had previously demonstrated [6] that exposure of normal chicks to aminophylline increases the 24-hydroxylase activity dramatically. Similar results have been obtained by Pedersen *et al.* [8], who used rat kidney mitochondria. In later studies we were able to show inhibition of 1 α -hydroxylase activity by renal mitochondrial protein kinase-catalysed phosphorylation of the ferredoxin [9,10]. The recent report by Siegel *et al.* [11] provided additional support for the finding that phosphorylation of ferredoxin is a likely regulatory mechanism. However, the role of the phosphorylated ferredoxin in the 24-hydroxylation reaction remained unknown.

Matocha & Waterman [12] reported in 1985 that the ox adrenal mitochondrial ferredoxin is encoded by nuclear genes yielding a precursor protein of 19 kDa in size which is processed to a mature form of 12 kDa upon uptake by mitochondria. Because antibodies to the ferredoxin were available to us, it seemed feasible to probe the role of synthesis of nascent ferredoxin in the hydroxylase activities and to test whether we could draw

Abbreviations used: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 24,25(OH)₂D₃, 24,25-dihydroxyvitamin D₃; 25(OH)D₃, 25-hydroxyvitamin D₃; poly(A)⁺, polyadenylated.

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correlations between the 'in vitro' translational capacity of isolated RNA and the measured hydroxylase activities. Therefore our efforts were directed at examining the activity of phosphorylated ferredoxin in reconstituted 1α - and 24-hydroxylase systems. We also used immunoprecipitation techniques to isolate newly synthesized nascent ferredoxin from a cell-free rabbit reticulocyte translational system programmed with poly(A)⁺ RNA prepared from chick kidney tissue. The results show that phosphorylation of the ferredoxin represents a post-translational regulation of the 1α - and 24-hydroxylases.

EXPERIMENTAL

Materials

Polyacrylamide electrophoresis reagents were purchased from Bio-Rad Laboratories. Aminophylline, guanidine thiocyanate, guanidium chloride and synthetic protein kinase inhibitor were obtained from Sigma. CsCl was purchased from Aldrich Chemicals. [³⁵S]Methionine (sp. radioactivity 1128 Ci/mmol) was purchased from du Pont/NEN Products and [5-³H]orotic acid (sp. radioactivity 20 Ci/mmol) was from Amersham Corp. Oligo(dT)-cellulose was obtained from Bethesda Research Laboratories. Nuclease-treated rabbit reticulocyte lysate was purchased from Promega Biotech and pre-stained molecular-mass markers were from Integrated Separation Systems. Pansorbin cells and the detergent Nonidet P40 were obtained from Calbiochem; Auto-fluor autoradiography enhancer was from National Diagnostics; Kodak XAR-5 films were from Winchester X-Ray Inc. Monospecific anti-ferredoxin was obtained as reported previously [13].

Animals

White Leghorn cockerel chicks, 1 day old, were made vitamin D-deficient by maintaining them on a rachitogenic test diet for 4 weeks. They were housed in the absence of u.v. light in a room illuminated only by a small incandescent lamp to enable the birds to feed. Normal birds were fed a vitamin D-replete diet until killed. Aminophylline was administered intraperitoneally to normal birds 3 h before they were killed [6] at a dose of 78 mg/kg body weight as a solution of 160 mg of aminophylline dissolved in 5 ml of 0.25 M-sucrose/15 mM-Tris acetate/0.1 mM-EDTA, pH 7.4. This vehicle was used to maintain consistency with previously published procedures [6]. Whole kidneys were removed from all animals killed by decapitation and frozen in liquid N₂ immediately.

Isolation of total RNA

Total RNA of kidney tissue was prepared by the guanidine thiocyanate/caesium chloride extraction method of Chirgwin *et al.* [14], with the exception that *N*-dodecylsarcosine was omitted. The crystallized RNA was collected by centrifugation and dissolved in 1 ml of double-distilled autoclaved water. Optical spectra of 20-fold diluted RNA were recorded in a microprocessed Beckman DU-40 spectrophotometer equipped with an Epson FX-80 printer. Concentrations of RNA were calculated from A_{260} given an A_{260} of 20 for a pure reference RNA at a concentration of 1 mg/ml.

Preparation of poly(A)⁺ RNA

Poly(A)⁺ RNA was prepared from total RNA by oligo(dT)-cellulose affinity chromatography as described by Aviv & Leder [15], with the exception that 10 mM-Tris/HCl, pH 7.4, was used as the elution buffer.

[³H]Orotate incorporation into poly(A)⁺ RNA

Groups of normal control and vitamin D-deficient birds were given 25 μ Ci of aqueous [5-³H]orotic acid (1.2 nmol) intraperitoneally 4 h before being killed. The normal birds receiving aminophylline were given the phosphodiesterase inhibitor as described above 1 h after orotic acid administration (3 h before being killed). Poly(A)⁺ RNA was isolated from the kidneys by oligo(dT)-cellulose chromatography of total RNA preparations as described above. The radioactivity incorporated into poly(A)⁺ RNA was determined by liquid-scintillation counting and expressed as a percentage of radioactivity (c.p.m.) in total RNA, which corrects for the variability of isotope incorporation in total RNA.

Cell-free translation with a rabbit reticulocyte-lysate system

Poly(A)⁺ RNA isolated from oligo(dT)-cellulose chromatography was used directly in the indicated amounts with nuclease-treated rabbit reticulocyte-lysate translation kit, the instructions provided with the kit being followed. [³⁵S]Methionine was included at a concentration of 1 μ Ci/ μ l of translation mixture. A 5 μ l aliquot from each translation reaction mixture was removed for determination of [³⁵S]methionine incorporation into trichloroacetic acid-precipitable proteins as described in the kit instructions.

Immunoprecipitation and electrophoresis

Nascent ferredoxin in total translation products was quantified by the method of Ivarie & Jones [16], as modified by DuBois *et al.* [17], *Staphylococcus aureus* Cowan A-strain cells (SAC, Pansorbin cells) being used. The immunoprecipitate-Pansorbin complexes were suspended in 50 μ l of Laemmli [18] SDS/PAGE sample buffer and heated at 100 °C for 5 min. The cells were pelleted by centrifugation and discarded. A portion of glycerol/Bromophenol Blue dye solution was added to the supernatant, which was subjected to electrophoresis for 8 h at 30 mA in an SDS/20%-acrylamide slab gel (Protean-II apparatus, Bio-Rad). One lane of pre-stained marker proteins was also included. After fixing for 1 h in ethanol/water/acetic acid (5:5:1, by vol.) the gels were soaked for 1 h in Auto-fluor (5%, v/v, in glycerol), then dried and used for autoradiography.

Autoradiography

Analysis was performed with XAR-5 films exposed to the dried slab gels in sealed cassettes at -70 °C for the indicated periods. The exposed films were developed by standard techniques. The band areas representing the ferredoxin in the autoradiograms were quantified with an LKB UltroScan XL laser densitometer with an on-board computer menu-driven program for integration and printing of results.

Preparation of mitochondrial ferredoxin and cytochrome P-450

The cytochrome was prepared from isolated mito-

chondria of kidneys obtained from chicks fed a normal diet for 4–5 weeks as described previously [10]. The cytochrome thus prepared was rendered free of detergent by hydroxyapatite chromatography as described by Gray & Ghazarian [19]. Ferredoxin was prepared according to published procedures [20].

Activity of phosphorylated ferredoxin

A 500 pmol portion of the ferredoxin was phosphorylated with 50 μ g of purified kidney mitochondrial protein kinase prepared as described previously [6]. The reaction mixture, in a total volume of 225 μ l, contained 175 μ M-ATP, 10 mM-magnesium acetate and 50 μ M-cyclic AMP in 20 mM-Mes buffer. The mixtures were incubated at 30 °C for the indicated time points then terminated by the addition of 15 μ l (1.5 μ g) of Sigma synthetic protein kinase inhibitor (Thr-Thr-Tyr-Ala-Asp-Phe-Ile-Ala-Ser-Gly-Arg-Thr-Gly-Arg-Arg-Asn-Ala-Ile-His-Asp) in water. The reaction mixtures were then divided into 120 μ l aliquots and each was reconstituted for hydroxylase activity in a total volume of 500 μ l by the addition of 25 pmol of cytochrome P-450 and other functional components as reported by Ghazarian & Yanda [10], except that 830 ng of the substrate 25(OH)[methyl-26(27)-³H]D₃ with a sp. radioactivity of 250 d.p.m./ng was used (4.1 μ M). The incubation time was 1 h at 30 °C. The reactions were quenched by the addition of 3.5 ml of methanol/chloroform (2:1, v/v) and radioactive products were extracted as described by Bligh & Dyer [20] for separation by reversed-phase h.p.l.c. on an IBM ODS 4.2 mm $\times$ 250 mm column using 80% (v/v) methanol in water at a flow rate of 1 ml/min. A total of 50 lml fractions were collected, dried in air, then counted for radioactivity at an efficiency of 60%. The elution of non-radioactive authentic metabolites as standards was monitored at 280 nm for the identification of the enzymic products. The amounts of products formed were calculated from the specific radioactivity of the substrate, given an extraction recovery of greater than 90% and a column recovery of about 75%.

Activity of mitochondrial cyclic AMP phosphodiesterase

Cyclic nucleotide phosphodiesterase activity was measured with sonicated mitochondrial extracts according to the procedure of Thompson & Appelman [21].

RESULTS

Recovery and purity of RNA

Although the method of Chirgwin *et al.* [14] for the isolation of RNA is satisfactory, the modified procedures we have used gave overall yields which compared favourably with those of Chirgwin *et al.* [14] and with the phenol/proteinase K procedure of Frazier *et al.* [22]. The recoveries of total RNA from the kidneys of the three groups of birds are shown in Table 1. Although the purity of the RNA preparations based on A_{260}/A_{280} ratios was the same for all animals, the quantity of RNA recovered from aminophylline-dosed birds was consistently greater than the amounts obtained from the remaining two groups. The application of oligo(dT)-cellulose affinity chromatography improved the purity of the separated poly(A)⁺ RNA fraction as determined from the A_{260}/A_{280} ratios.

Table 1. Recovery and purity of chick kidney RNA

Animal status	n*	RNA (mg/g of tissue)	A_{260}/A_{280}	
			Total RNA	Poly(A) ⁺ RNA†
Normal control	10	1.54 $\pm$ 0.1	1.74	1.92‡
Aminophylline-dosed	5	2.32 $\pm$ 0.2	1.72	2.02
Vitamin D-deficient	5	1.19 $\pm$ 0.1	1.71	2.01

* Number of times RNA was prepared.
† After oligo(dT)-cellulose chromatography.
‡ No significant difference within the groups at $P < 0.05$ (*t* test).

[³H]Orotate incorporation into poly(A)⁺ RNA

In vitamin D deficiency the 1 α -hydroxylase is predominant and little or no 24-hydroxylase activity is detected. On the other hand, the predominance of the 24-hydroxylase in the normal bird kidney has been shown to be subject to severalfold activation by administration of aminophylline. In an effort to correlate these observations with nascent RNA synthesis, poly(A)⁺ RNA was isolated from the respective birds given [³H]orotic acid. The extent of uridine incorporation into poly(A)⁺ RNA was determined and expressed as a percentage of uridine incorporation into total RNA. The results are shown in Table 2. Although total RNA synthesis in vitamin D-deficiency is not significantly different from that of the normal controls, it is clear that, in the kidneys of animals given aminophylline, there is a significant increase in transcriptional activity, although this may simply reflect selective amplification of existing RNAs. It is important to note that the steady-state concentration of the poly(A)⁺ RNA pool in vitamin D deficiency does not seem to be different from that in the normal bird when expressed as c.p.m. per mg of RNA.

Fig. 1 shows the ability of the isolated poly(A)⁺ RNA to support *in vitro* cell-free translation in rabbit reticulocyte lysates. The increase in methionine incorporation by the poly(A)⁺ RNA of aminophylline-dosed birds, as determined by trichloroacetic acid precipitable [³⁵S]methionine from total translation products, may reflect increases in translation of specific RNAs only or enhanced encoding of proteins rich in methionine. In contrast, either (1) the RNA pool in vitamin D deficiency is not being totally translated or (2) the translation

Table 2. Incorporation of [³H]orotate into kidney RNA

Animal status	Uridine incorporation into poly(A) ⁺ RNA (% of that into total RNA)	10 ⁻³ $\times$ Radioactivity [c.p.m./mg of poly(A) ⁺ RNA]
Normal control	13.5 $\pm$ 3.0	3.34
Vitamin D-deficient	15.1 $\pm$ 2.0	2.84
Aminophylline-dosed	20.3 $\pm$ 5.0	4.39*

* The aminophylline-dosed group was significantly different from other groups at $P < 0.02$ (based on *t* test).

Fig. 1. [³⁵S]-Methionine incorporation in cell-free translations programmed with RNA of normal, aminophylline-dosed and vitamin D-deficient bird kidney poly(A)⁺ RNA

Bars with no RNA ('0') represent translations by rabbit-reticulocyte-lysate endogenous message.

products in deficiency contain less methionine than those synthesized by poly(A)⁺ RNA isolated from normal or aminophylline-dosed chicks.

Immunoprecipitation of ferredoxin

The densitometric analyses of the autoradiograms of immunoprecipitated ferredoxin from newly synthesized [³⁵S]methionine-labelled cell-free translation products (Fig. 2) are shown in Table 3. It is clear that only in vitamin D deficiency is there an accentuated translation of ferredoxin. Since the steady-state concentration of the poly(A)⁺ RNA pool in vitamin D deficiency is not different from that in the normal animal (see Table 2), it would be reasonable to conclude that the poly(A)⁺ RNA pool in vitamin D deficiency is preferentially enriched with the ferredoxin message. Furthermore, the increased translational activity in the aminophylline-dosed birds must represent ferredoxin-unrelated synthesis. It should be noted that, when the immunoprecipitation is conducted in the presence of exogenous antigen, the radioactivity incorporated into the ferredoxin cannot be detected (see the footnote to Table 3). On the basis of the relative mobilities of the marker proteins, a calculated molecular mass of 12–13 kDa is obtained for the immunoprecipitated ferredoxin.

Modulation of the hydroxylases by phosphorylation

Fig. 3 shows the elution characteristics of authentic vitamin D metabolites and of the vitamin D metabolites extracted from the reconstitution assays in which the

Fig. 2. Densitometric tracing of autoradiograms of immunoprecipitated nascent ferredoxin from [³⁵S]methionine-labelled cell-free translation products which were separated by SDS/20 %-PAGE

Exposure time at -70°C was 5 weeks. (a) Normal; (b) aminophylline-dosed; (c) vitamin D-deficient; (d) vitamin D-deficient ($8\text{ }\mu\text{g}$ of exogenous ferredoxin was added during immunoprecipitation).

Table 3. Densitometric analysis of the autoradiograms of ferredoxin immunoprecipitated from kidney RNA-directed 'in vitro' translation

Translation RNA source	Panel (see Fig. 2)	Peak position (mm)	Relative area (absorbance units $\times$ mm)
Normal control	(a)	109.5	0.20
Aminophylline-dosed	(b)	108.5	0.24
Vitamin D-deficient	(c)	109.4	0.38
Vitamin D-deficient*	(d)	ND†	ND

* Inclusion of $8\text{ }\mu\text{g}$ of exogenous antigen during the immunoprecipitation experiments causes the complete disappearance of the radioactivity in the autoradiograms in the region of ferredoxin peak position; therefore, no relative-area value can be calculated.

†ND, not detectable.

Fig. 3. Chromatography of authentic vitamin D metabolites on an IBM ODS column

The solvent system was methanol/water (4:1, v/v) and the flow rate was 1 ml/min. Amounts of metabolites run were as follows: 24,25(OH)₂D₃, 172 ng; 1 α ,25(OH)₂D₃, 430 ng; 25(OH)D₃, 216 ng.

effects of degree of phosphorylation of ferredoxin on the production of 1 α ,25(OH)₂D₃ and 24,25(OH)₂D₃ were evaluated. The quantification of these products is shown in Table 4. The data indicate a reciprocal relationship between the 1 α -hydroxylase activity and the 24-hydroxylase activity reconstituted after phosphorylation of the ferredoxin with protein kinase. It should be noted here that, although we do not know the precise relationship between the two *P*-450 cytochromes which are present in the preparation we have used in these reconstitution assays, we have, however, demonstrated that they share common properties (M. L. Mandel, B. Moorthy & J. G. Ghazarian, unpublished work). There is no doubt that, at least in the pig, the two cytochromes in kidney mitochondria with their characteristic activities can be clearly separated by ion-exchange chromatography [19]. As shown in Table 4, a common ferredoxin functions as the sole specific electron-transfer protein necessary for the hydroxylation reactions. However, the positional specificity of hydroxylation in the structure of the substrate 25(OH)D₃ depends on the phosphorylation state of the ferredoxin. The decrease in the 1 α -hydroxylase activity and the concomitant increase in the 24-hydroxylase activity parallel the phosphorylation reaction in a time-dependent fashion. After 2 min of

Table 4. Ability of ferredoxin to support hydroxylase activities as a function of its extent of phosphorylation

Incubation conditions were the same as those described in the Experimental section at a uniform ferredoxin/cytochrome *P*-450 molar ratio of 20:1. Under similar conditions, but at a substrate concentration of 0.06 μ M and over a phosphorylation period of 10 min, the 1 α -hydroxylase activity is totally lost. Also, at a ferredoxin/cytochrome *P*-450 ratio of 200:1 and over a phosphorylation period of 4 min, the 24-hydroxylase activity was 2.34 mol/mol of cytochrome *P*-450. Each value represents the mean of a minimum of four separate determinations.

Period of phosphorylation (min)	24-Hydroxylase activity (mol/mol of cytochrome <i>P</i> -450)	1 α -Hydroxylase activity (mol/mol of cytochrome <i>P</i> -450)
0	1.46	0.576
2	1.92	0.616
5	1.80	0.576
15	1.80	0.500
30	1.50	0.424
60	1.43	0.440

phosphorylation immediately preceding the reconstitution of hydroxylase activity at a cytochrome/ferredoxin molar ratio of 1:20 the phosphorylated ferredoxin causes a 32% increase in the production of 24,25(OH)₂D₃. This increase in the 24-hydroxylase activity can be as great as 60% over control, depending upon the quantity of ferredoxin used and the duration of phosphorylation before reconstitution of activity. Although the production of 24,25(OH)₂D₃ is stimulated in the presence of the phosphorylated ferredoxin, the activity of the 1 α -hydroxylase is gradually lost and this loss can be as great as 100% [10]. Although we see a decrease in the 24-hydroxylase activity after 10 or 15 min of phosphorylation, the final 24-hydroxylase activity does not fall below control values. In contrast, the 1 α -hydroxylase activity continues to decrease with time. These results are consistent with our recent findings

Table 5. Kidney mitochondrial cyclic AMP phosphodiesterase activity under various dietary conditions

Mitochondria were isolated from the kidneys of the respective birds and sonicated for 2 min, then centrifuged at 100 000 *g* for 30 min. The supernatants were used as the enzyme source. Incubation conditions were the same as those described by Thompson & Appelman [21].

Dietary treatment	Activity (ng of cyclic AMP hydrolysed · mg of mitochondrial protein ⁻¹)
Normal, 1 day old	0.92 $\pm$ 0.09
Normal, 6 weeks old	1.09 $\pm$ 0.14
Vitamin D-deficient	0.75 $\pm$ 0.12
Calcium deprivation, 1 day	0.98 $\pm$ 0.07
Calcium deprivation, 3 days	0.64 $\pm$ 0.12
Aminophylline-dosed	2.09 $\pm$ 0.11

that one serine and one threonine residue in the ferredoxin are phosphorylated, although the order of their phosphorylation is not known [23]. Therefore the phosphorylation of ferredoxin appears to be responsible for the suppression of the 1α -hydroxylase and stimulation of the 24-hydroxylase. This interpretation is consistent with the reported results of Monnier *et al.* [24] concerning the role of phosphorylated ferredoxin in the activities of the adrenal mitochondrial steroidogenic *P*-450 cytochromes.

Finally, the data presented in Table 5 show the effects of dietary changes on the kidney mitochondrial cyclic nucleotide phosphodiesterase activity. It can be seen that, regardless of the dietary manipulations, the measured phosphodiesterase activities did not seem to change significantly unless the birds were dosed with aminophylline, an agent which also causes a severalfold increase in the 24-hydroxylase activity.

DISCUSSION

Mitochondria of kidney tissue contain, in addition to the normal enzymes required for oxidative metabolism, and especially in the proximal-tubular regions of the nephron [25], enzymes which function in the hydroxylation of vitamin D metabolites [26]. Of these enzymes, the most extensively studied for their functional importance are the 1α -hydroxylase and the 24-hydroxylase, which use $25(\text{OH})\text{D}_3$ as their common substrate. These enzymes are multiple complexes, each requiring several electron-transferring intermediary proteins for the ultimate hydroxylation of $25(\text{OH})\text{D}_3$ to the corresponding product, coincident with the reduction of one atom of molecular oxygen to water [7,27]. Each of these enzymes is composed of a ferredoxin reductase, ferredoxin and cytochrome *P*-450 [3,6,28–30]. There has been no direct evidence that the amounts of these proteins within the mitochondrion affect the functional capacity of the kidney in maintaining Ca^{2+} homeostasis.

The mitochondrion contains DNA, RNA and the biochemical machinery necessary for transcriptional and translational processes. However, most of the mitochondrial proteins are encoded by nuclear genes, assembled in the cytoplasm and subsequently imported into the mitochondrion. Adrenodoxin, for example, which bears relevance to our work because it is the ferredoxin component of the adrenal mitochondrial steroidogenic mono-oxygenases, is encoded by nuclear DNA, is assembled as a larger precursor molecule [31] and is processed to the mature form of molecular mass 12 kDa during its import into mitochondria. The processing involves the cleavage of a 58-residue *N*-terminal signal peptide and a 14-residue *C*-terminal peptide that has a molecular mass of 1 573 Da [32]. There is no evidence to suggest the presence of adrenodoxin precursor molecules within adrenal mitochondria. In recent studies Okamura *et al.* [33] have described the occurrence of several distinct adrenodoxin mRNA species which are grouped into two sets. Only one common mature adrenodoxin sequence is encoded by both sets of mRNA, but one set is 10-fold more abundant than the other.

In the present study we have examined the effects of vitamin D deficiency and aminophylline administration to normal chicks on the '*in vitro*' activity of mRNA isolated from the respective kidneys. We find that the

amount of nascent ferredoxin synthesized in vitamin D deficiency is significantly greater than the synthesis in lysates programmed with mRNA of normal birds given aminophylline, despite the latter having the ability to catalyse a greater extent of methionine incorporation into total translational products. It would appear, therefore, that the RNA isolated from the kidneys of vitamin D-deficient birds is preferentially enriched with ferredoxin message, whereas the RNA of the treated birds is not. The greater level of methionine incorporation into the translations programmed with the latter RNA seems to be mostly unrelated to ferredoxin.

We first became aware of ferredoxin as a potential focus of regulation of the renal hydroxylases soon after we were able to demonstrate the presence of protein kinase activity in isolated kidney mitochondria. Those studies stemmed from our recognition that agents which alter tissue cyclic nucleotide concentrations also alter the measured hydroxylase activities *in vitro* [34–36]. Our first attempt to study the role of phosphorylated ferredoxin in vitamin D metabolism was confined to the 1α -hydroxylation of $25(\text{OH})\text{D}_3$. In 1985 we reported [10] that the phosphorylated ferredoxin failed to support the 1α -hydroxylation of $25(\text{OH})\text{D}_3$ in an '*in vitro*' reconstituted system containing cytochrome *P*-450 isolated from kidney mitochondria of vitamin D-deficient birds. Being aware from our earlier studies and from others [6,37] that the activity of the 24-hydroxylase in normal birds can be selectively stimulated by aminophylline, we asked whether this was a result of increased ferredoxin concentration in the renal mitochondrion. The results of such studies, which are reported here, caused a considerable confusion at first when we discovered that there was no change in nascent ferredoxin synthesis by the poly(A)⁺ RNA isolated from the aminophylline-dosed birds, despite the severalfold increase in the 24-hydroxylase activity in similarly treated birds. These observations and those reported by Siegel *et al.* [11] and by Monnier *et al.* [24] prompted us to undertake two additional studies. First, we examined the effect of phosphorylation of ferredoxin on the activity of cytochrome *P*-450 prepared from the kidneys of normal birds, and, secondly, we evaluated the renal mitochondrial cyclic nucleotide phosphodiesterase activity in normal birds, aminophylline-dosed birds, in vitamin D deficiency and in calcium deprivation. The results are shown in Tables 4 and 5. Clearly, the phosphorylation of ferredoxin increases the 24-hydroxylase activity, whereas it causes a gradual loss of 1α -hydroxylase activity. Although the data shown in Table 5 offer only preliminary evidence to what we believe is an essential part of the 1α -hydroxylase system, it is, nevertheless, reasonable to suggest that, in order to maintain an active 1α -hydroxylase, it is necessary that the phosphorylation of the ferredoxin is prevented, possibly through the activation of a phosphatase by a cyclic AMP-dependent protein kinase. This would be consistent with the generally observed increases in cellular cyclic AMP output concurrent with increases in 1α -hydroxylase activity [34,35]. Conversely, the aminophylline-induced increase in the mitochondrial phosphodiesterase activity, which coincides with a large increase in 24-hydroxylase activity, must be associated with the phosphorylation of ferredoxin. It is necessary to conclude, therefore, that the phosphorylation of ferredoxin must proceed via a cyclic nucleotide-independent kinase. It should be noted that

Scheme 1. Model illustrating the various relationships between the components of the 1 α - and 24-hydroxylases, depicting the role of phosphorylated ferredoxin and protein kinases

Abbreviations: P-450_{1 α} , 1 α -hydroxylase cytochrome P-450; P-450₂₄, 24-hydroxylase cytochrome P-450; F_p, flavo-protein; F_x, ferredoxin; Ca-kinase, Ca²⁺-activated protein kinase.

although aminophylline is used *in vitro* as an inhibitor of phosphodiesterase activity, its effects *in vivo*, especially in renal mitochondria, may be complex. Its uses range from muscle relaxant to a diuretic agent. Finally, the noted increase in the 24-hydroxylase activity by aminophylline is not likely to be due to a chemical change in the cytochrome P-450 moiety of the enzyme, because the concentration of the cytochrome in kidney mitochondria does not change in response to dietary manipulations [1,10], nor does its activity change with phosphorylation [10]. Scheme 1 summarizes the above relationships. We would like to suggest that the non-phosphorylated ferredoxin, which can support both 1 α - and 24-hydroxylase activities [19,28], becomes upon phosphorylation a more efficient electron carrier for the 24-hydroxylase, but less effective in supporting the 1 α -hydroxylation reaction. Therefore, in vitamin D deficiency, where the need for 1,25(OH)₂D₃ is great, there is an accentuated synthesis of the regulatory component of the 1 α -hydroxylase (the ferredoxin) in a futile effort (no circulating substrate in deficiency) to generate the critical metabolite necessary for the restoration of calcium homeostasis.

Driscoll & Omdahl [38] have recently reported the presence, in porcine kidney mitochondria, of two separate ferredoxins with molecular masses of 11.5 and 13 kDa. Although we could not definitively ascertain by immunoprecipitation and SDS/PAGE electrophoresis whether two separate ferredoxins are similarly produced in the cell-free translation system we have used, our

results, nonetheless, would not support the existence of two ferredoxins. In this context, it should be noted that Hiwatashi *et al.* [39] were able to demonstrate that the heterogeneity of purified ferredoxins was a result of proteolytic hydrolysis during the purification of a single parent molecule. Since the fully processed renal mitochondrial ferredoxin has a molecular mass of 11.9 kDa [3], the '*in vitro*' RNA-directed translation and immunoprecipitation of the ferredoxin with a molecular mass of 12–13 kDa we have reported in the present study may indeed represent the fully mature ferredoxin, unlike the adrenal studies undertaken by Matocha & Waterman [12].

Although it remains to be shown whether the ferredoxin is phosphorylated *in situ*, there is little doubt that it is indeed phosphorylated in the intact renal cell, as shown by '*in vitro*' tissue-slice experiments [11]. Furthermore, this phosphorylation is greatly diminished upon exposure of the tissue to parathyroid hormone, and is accompanied by an increase in 1 α -hydroxylation of 25(OH)D₃, which is a well-recognized biological response of the kidney to the effect of the hormone. Our observations collectively would seem to suggest that the structural association of the ferredoxin with the 1 α -hydroxylase cytochrome P-450 is altered upon phosphorylation of the ferredoxin. This structural alteration may bring about a distortion in the catalytic interaction of the cytochrome with the ferredoxin, thus favouring the shuttling of electrons to a juxtapositioned cytochrome with a site specificity for the hydroxylation of the side chain of 25(OH)D₃ at position C-24.

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